Dawn and dusk

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Circadian rhythmicity in constant light in mice lacking functional *Per* or *Cry* genes

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ABSTRACT

Mutations in each of the genes *mPer1, mPer2, mCry1* and *mCry2* separately cause the circadian system to deviate from that in wild type mice, either in period length in constant darkness (DD) or in circadian phase resetting in response to brief light pulses. Differences between the mutant strains have inspired the hypothesis that the duality of circadian genes (2 *mPer* and 2 *mCry* genes involved) is related to the existence of two components in the circadian oscillator (Daan et al. 2001). From this theory the robust prediction was derived that the circadian period lengthens under constant illumination (LL) with increasing light intensity in mPer1 and *mCry1* mutant mice, while it shortens in *mPer2* and *mCry2* mutants. We investigated these predictions in mice lacking functional *mPer* or *mCry* genes in constant illumination. Results for mPer mutant mice are in agreement with data reported earlier by Steinlechner et al (2002a), and with the predictions from the model. Changes in period (τ) observed appear unrelated to the overall reduction of activity with increasing light intensity which was observed in both knockout strains as well as in wildtype mice. *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ knockout mice consistently increased τ with increasing light intensity, such that the large τ difference between *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice in DD is retained under constant illumination. To quantify expression of circadian rhythmicity in running wheel behavior, we calculated the Signal to Noise Ratio (SNR). Opposite effects of increasing illumination on SNR were demonstrated in *mPer1* and *mPer2* mutant mice. In *mPer1* mutant mice circadian rhythm SNR is more strongly reduced by constant light than in wild types. In *mPer2* mutant mice increasing light intensity initially enhances SNR. In the *mCry* mutants light effects on SNR are similar to wild type. Hence, the mutations of *mPer1* and *mPer2* have opposite effects on the influence of constant light on the circadian system, while the deletions of *mCry1* and *mCry2* cause opposite effects on circadian period independent of the light intensity. Deceleration of the circadian system by light in both *mCry1* and *mCry2* mutants violates the predictions from the model, which therefore has to be modified with respect to the *mCry* genes, but not to the *mPer* genes.

INTRODUCTION

Continuous illumination (LL) has two classic effects on the expression of circadian rhythms, on the degree of rhythmicity and on the circadian period. LL, especially of high light intensity, generally causes suppression of rhythmicity (Aschoff 1960; Daan and Pittendrigh 1976b). It further tends to decelerate circadian rhythms in mammals. The increasing cycle length with increasing levels of constant illumination was originally considered to be specific for night-active animals (Aschoff 1960; Aschoff 1964). On the basis of accumulating mammalian data Aschoff (1979) later changed this rule into the generalization that all mammals, diurnal as well as nocturnal, lengthen the circadian period (τ) with increasing intensity of illumination. These ubiquitous effects of light have so far rarely been considered in the context of investigations on the molecular biology of circadian rhythms. Yet, the responses may be of considerable interest. Two studies have reported exceptional LL phenotypes in animals with mutant circadian genes: recovery from – rather than induction of - arrhythmicity in LL in *mClock* mutant mice (Spoelstra et al. 2002) and acceleration rather than deceleration of the circadian cycle in LL in *mPer2* mutant mice (Steinlechner et al. 2002a). These results suggest that it may be worthwhile to collect more information on rhythmicity in LL in circadian gene mutants. In particular, a recent hypothesis on the role of *mPer1, mPer2, mCry1* and *mCry2* in accelerating and decelerating responses to light in subcomponents of the circadian oscillator yields specific predictions for the effect of gene deletions in these responses (Daan et al. 2001). In this study we set out to test these predictions.

METHODS

The experiment included 8 *mPer1*^{Brdm1}, 8 *mPer2*^{Brdm1}, 8 wild type mice (C57BL/6 x 129 SvEvBrd genetic background); 6 *mCry1*$^{-/-}$, 8 *mCry1*$^{-/-}$ and 8 wild type mice (C57BL/6 x 129ola background). The generation of the mutants has been described by van der Horst et al. (1999) for the *mCry* knockout strains and by Zheng et al. (1999) for the *mPer* mutants. Animals were housed individually in 25x25x40 cm cages, with food and water *ad libitum*. Spontaneous locomotor activity was recorded with running wheels (Ø 14 cm) connected to an Event Recording System (ERS) storing wheel revolutions in 2 minute intervals. Temperature was maintained at 23 ± 1 °C throughout the entire experiment.

All cages were placed in our Activity Controlled Illumination System (ACIS). This is a custom designed experimental setup with 24 compartments (75x50x70 cm), in which any light intensity between 0 – 1500 Lux can be offered. The compartments are each provided with an overhead battery of 2 fluorescent tubes (Philips fluotone TLD85W/83°) illuminating the cages through an opaque glass partition. Directly

above this partition a horizontal shutter closes off the light sources by a computer-controlled electric motor, to ensure continuous control of light intensity without spectral change. Light intensity is continuously recorded at the bottom of each compartment. The shutter is operated as long as the computer senses a difference between its preset light intensity and the intensity recorded by the sensor. Fans mounted in light locks in the back of each compartment take care of ventilation and thereby prevent temperature change as a consequence of different illumination intensity. The system can either provide a light intensity profile, *e.g.*, mimicking twilights according to any latitudinal specification, or as was used here, simply provide continuous illumination of any intensity.

All mice were entrained to LD 12:12 (L 1000 Lux) for 14 days, and then exposed successively for 15 days to DD, 10 days to LL 1 Lux, 17 days to LL 200 Lux, 14 days to LL 1000 Lux, and 14 days in LL 10 Lux. All mice were then re-entrained to LD 12:12 for 42 days and then exposed to constant illumination in 14 day sections with consecutive light intensities of 1000, 100, 10, and 1 Lux.

To assess period length, individual activity data from each section of the record were subjected to chi-square periodogram analysis (Sokolove and Bushell 1978). We further evaluated the effects of different intensities in constant illumination on the amount of activity (average number of wheel revolutions per hour) and on the degree of rhythmicity in the pattern recorded. For this last purpose, we determined the Signal to Noise Ratio (SNR) for the most prominent rhythm in the activity pattern. The SNR has been used to quantify the strength of a circadian rhythm previously (White et al. 1992; Ruf 1999) and is calculated by dividing the variance of the signal by the variance of the noise. Here the signal is the averaged activity pattern of a circadian rhythm with a period between 20 and 30 h detected by periodogram analysis.

RESULTS

Figure 9.1 (*mPer* mutant mice) and 2 (*mCry* knockout mice) show actogram examples representative for the six genotypes analyzed. Circadian rhythms in the $mPer1^{Brdm1}$ mouse decreased in period length in DD and lengthened in LL. Circadian rhythmicity gradually disappeared in $mPer1^{Brdm1}$ mice when exposed to bright light, and was restored with decreasing light intensity. Opposite trends in circadian period length and rhythmicity are observed in $mPer2^{Brdm1}$ mice. These mice lost rhythmicity in low light intensity and regained their circadian rhythm with shortened period length in bright light. The $mCry1^{-/-}$ mice shortened circadian period length in DD, and lengthened it with increasing light intensity. Circadian period in $mCry1^{-/-}$ lengthened in DD relative to LD 12:12, and lengthened even more in constant light. In all three *mCry* strains rhythmicity was reduced but preserved in both DD and LL.

Consecutive individual τ values for all genotypes and corresponding light intensity are

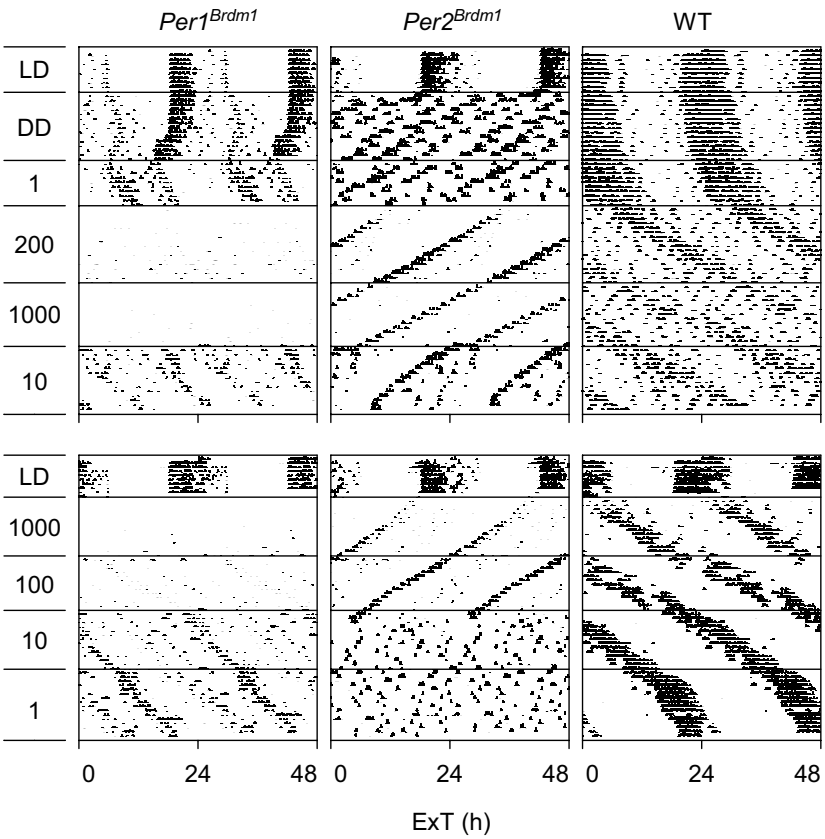

Figure 9.1 Actogram examples of *mPer* strains and wildtype mice in light with increasing and decreasing intensity. Horizontal lines delimit LL light intensities (Lux) denoted on the left.

plotted in figure 9.3. Figure 9.4 shows the average τ values for the whole 0-1000 Lux range (for 100-200 Lux individual values were first averaged). The actual values are listed in table 9.1. Wild type mice increased their period length from 24.1 h in DD to 25.9 h in 1000 Lux. *mPer1*^{Brdm1} mice lengthened their circadian period more strongly from 24.2 h in DD to 27.8 h in 1000 Lux. Few animals remained rhythmic during high light intensity. Nonetheless the difference from wild type was significant ($p<0.05$) at 100 Lux exposure. All *mPer2*^{Brdm1} mice shortened circadian period length when exposed to constant light, with the shortest period in 1 and 10 Lux. Period length values in *mPer2*^{Brdm1} mice were significantly shorter than in wild type or *mPer1*^{Brdm1} mice in all LL intensities. The SNR of the most prominent rhythm detected between 20 and 30 h in DD was close to zero in *mPer2*^{Brdm1} mice. LL restored SNR values in mPer2Brdm1 mice. Only when continuously exposed to 1000 Lux SNR values slightly decreased again (figure 9.4). SNR values in *mPer1*^{Brdm1} mice were low in DD and

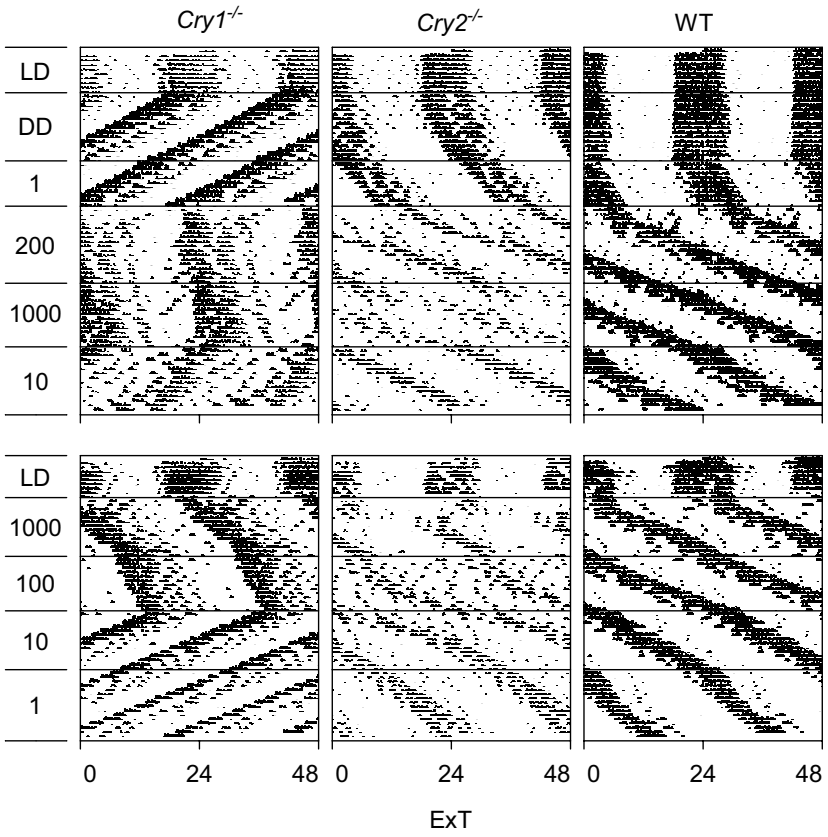

Figure 9.2 Actogram examples of *mCry* strains and wildtype mice in light with increasing and decreasing intensity. Horizontal lines delimit LL light intensities (Lux) denoted on the left.

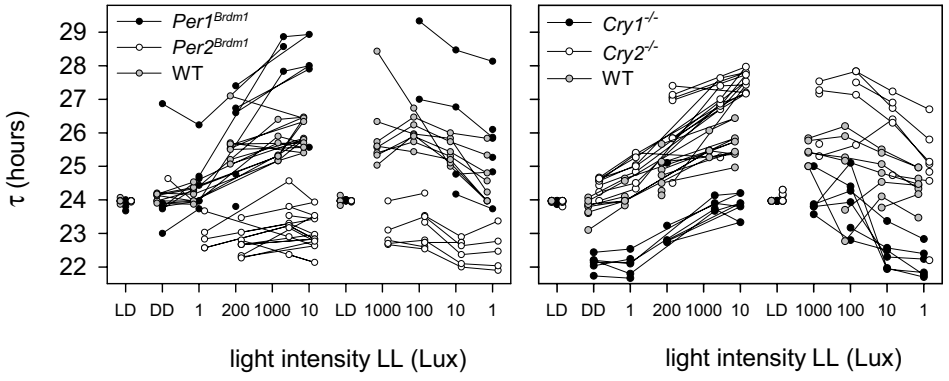

Figure 9.3 Individual τ values in constant illumination with increasing and decreasing intensity.

decreased further with increasing LL light intensity. Wildtype mice were on average more rhythmic than *mPer* mutant mice, with SNR values gradually decreasing with increasing light intensity.
The average activity level (wheel revolutions * h^{-1}) in entrainment was reduced in *mPer2*^{Brdm1} mice and even more reduced in *mPer1*^{Brdm1} mice compared to wild type mice (Figure 9.4). This difference was retained in DD and in LL at all light intensities. All

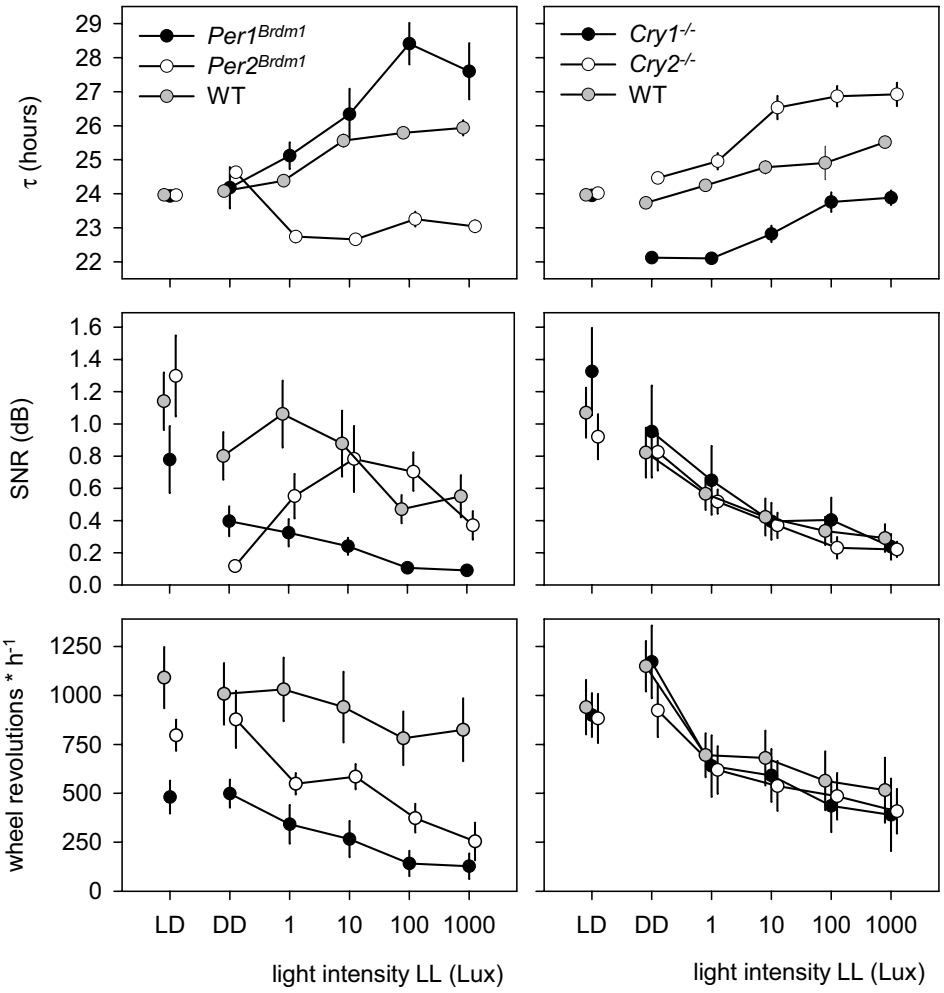

Figure 9.4 Upper graphs: τ values averaged over equal light intensities during the course of the experiment. Error bars indicate 1 SEM. Middle graphs: average activity level in wheel revolutions/h with increasing light intensity. Error bars indicate 1 SEM. Lower graphs: level of rhythmicity expressed by the Signal to Noise Ratio (SNR). A stable rhythm is reflected by a high signal to noise ratio.

three *mPer* strains showed an overall decrease in hourly activity. The sharpest decrease was observed in *mPer2*^{Brdm1} mice.

All *mCry*$^{-}$ strains lengthened their circadian period with increasing light intensity (Figure 9.4). For all light intensities, *mCry1*$^{-/-}$ mice had a significantly shorter, and *mCry2*$^{-/-}$ mice had a significantly longer period than wild type control mice, corresponding to the difference in DD. SNR values for all *mCry* strains were on average highly similar across all LL intensities, and gradually decreased with increasing light intensity.

Average activity levels were equal in LD, DD and in all LL light intensities in all *mCry* strains. Increasing light intensity increasingly suppressed activity levels.

Table 9.1 Circadian period length (h) in mutant mice when exposed to constant illumination with increasing intensity. Pairwise comparisons are made by Mann-Whitney Rank Sum Tests.

	mPer1^{Brdm1}			*mPer2*^{Brdm1}			*WT*			*mPer1*^{Brdm1} *WT*	*mPer2*^{Brdm1} *WT*	*mPer1*^{Brdm1} *mPer2*^{Brdm1}
	Mean	SE	n	Mean	SE	n	Mean	SE	n	*p*	*p*	*p*
Activity (wheel revolutions / h)												
DD	24.2	0.6	6	24.6	-	1	24.1	0.0	8	<0.05	-	-
1 Lux	25.1	0.4	8	22.7	0.2	7	24.4	0.2	8	ns	<0.001	<0.001
10 Lux	26.3	0.7	6	22.7	0.2	8	25.6	0.2	8	ns	<0.001	<0.001
100 Lux	28.4	0.6	3	23.3	0.2	8	25.8	0.1	8	<0.05	<0.001	<0.05
1000 Lux	27.6	0.8	4	23.0	0.2	8	25.9	0.2	8	ns	<0.001	<0.01

	mCry1$^{-/-}$			*mCry2*$^{-/-}$			*WT*			*mCry1*$^{-/-}$ *WT*	*mCry2*$^{-/-}$ *WT*	*mCry1*$^{-/-}$ *mCry2*$^{-/-}$
	Mean	SE	n	Mean	SE	n	Mean	SE	n	*p*	*p*	*p*
Activity (wheel revolutions / h)												
DD	22.1	0.1	6	24.5	0.1	8	23.7	0.1	8	<0.001	<0.001	<0.001
1 Lux	22.1	0.1	6	25.0	0.2	8	24.2	0.1	8	<0.001	<0.05	<0.001
10 Lux	22.8	0.2	6	26.5	0.3	8	24.8	0.2	8	<0.001	<0.01	<0.001
100 Lux	23.8	0.3	6	26.9	0.3	8	24.9	0.5	7	ns	<0.01	<0.001
1000 Lux	23.9	0.2	5	26.9	0.3	7	25.5	0.2	6	<0.01	<0.01	<0.01

Table 9.2 Running wheel activity (revolutions * h^{-1}) in mutant mice when exposed to constant illumination with increasing intensity. Pairwise comparisons are made by Mann-Whitney Rank Sum Tests.

	mPer1^{Brdm1}			*mPer2*^{Brdm1}			*WT*			*mPer1*^{Brdm1} *WT*	*mPer2*^{Brdm1} *WT*	*mPer1*^{Brdm1} *mPer2*^{Brdm1}
	Mean	SE	n	Mean	SE	n	Mean	SE	n	*p*	*p*	*p*
Activity (wheel revolutions / h)												
DD	498	71	8	878	147	8	1008	157	8	0.005	ns	0.01
1 Lux	342	99	8	549	55	8	1031	162	8	<0.005	<0.05	ns
10 Lux	266	93	8	585	65	8	941	180	8	0.005	ns	<0.05
100 Lux	142	64	8	373	73	8	781	137	8	<0.005	<0.05	<0.05
1000 Lux	128	65	8	254	96	8	825	161	8	<0.005	<0.05	ns

	mCry1$^{-/-}$			*mCry2*$^{-/-}$			*WT*			*mCry1*$^{-/-}$ *WT*	*mCry2*$^{-/-}$ *WT*	*mCry1*$^{-/-}$ *mCry2*$^{-/-}$
	Mean	SE	n	Mean	SE	n	Mean	SE	n	*p*	*p*	*p*
Activity (wheel revolutions / h)												
DD	1172	186	6	924	136	8	1149	129	8	ns	ns	ns
1 Lux	639	157	6	619	121	8	695	113	8	ns	ns	ns
10 Lux	591	135	6	537	127	8	680	140	8	ns	ns	ns
100 Lux	435	134	6	485	118	8	564	150	8	ns	ns	ns
1000 Lux	390	185	6	408	114	8	516	166	8	ns	ns	ns

DISCUSSION

Circadian period

Circadian period lengths for *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mutant mice in increasing and decreasing light intensities in our study were similar to those reported by Steinlechner et al. (2002a). Our results confirm their finding that period length shortens in *mPer2*^{Brdm1} in constant light relative to DD. While general in (diurnal) birds, this accelerating response to constant light is unique among mammals, where so far all species measured exhibit an increase in circadian period in LL (Aschoff 1979). The shortening of τ in *mPer2*^{Brdm1} mice in two studies is a remarkable confirmation of the prediction generated by the two-component model (Daan et al. 2001). In our study there appeared to be no further shortening of the circadian cycle with increasing intensity beyond 1 Lux in the *mPer2*^{Brdm1} strain as was observed by Steinlechner et al. (Steinlechner et al. 2002a). It is of interest that the circadian acceleration is accompanied by a decrease in activity level, where all other strains decelerate in combination with a decrease in activity level. There is a rather general negative

association between the amount of activity and circadian period (Aschoff 1960; Aschoff et al. 1973; Turek 1989). This may be caused by additional variables, such as testosterone titers (Daan et al. 1975) acting on both activity and the circadian system. It may also be due to a feedback effect from activity on the pacemaker. The present results demonstrate that the period shortening in LL can not be attributable to such feedback. The extra lengthening in circadian period in *mPer1*^{Brdm1} mice and the shortening in circadian period in *mPer2*^{Brdm1} mice suggests distinct roles for the *mPer1* and *mPer2* gene in accelerating and decelerating the circadian system, respectively.
In contrast to the two *mPer* mutant strains the *mCry* mutant mice express their differences in period length equally under different intensities of constant illumination. The increasing τ values both in *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ with increasing LL light intensity refute the prediction concerning the mCry genes derived from the two-component theory proposed by Daan et al. (2001). Apparently neither *mCry* gene separately has to be functional for the deceleration response to continuous light.

Activity

The activity level in all strains tested decreased with increasing light intensity. Activity levels in all three *mPer* genotypes in DD were similar to the entrainment situation. Activity levels in *mPer1*^{Brdm1} and *mPer2*^{Brdm1} mice in all light intensities were lower than in wild type control mice. The absence of functional *mPer1* most severely reduces locomotor activity. Constant light suppressed locomotor activity evenly in *mPer1*^{Brdm1} and wild type, but most severely in *mPer2*^{Brdm1}.
Activity levels in *mCry1*$^{-/-}$ and *mCry2*$^{-/-}$ mice were quite similar to those of wild type control mice in all photic conditions including LD and DD. In agreement with results obtained by Mrosovsky (2001), activity levels in all three genotypes were increasingly and evenly reduced by LL with increasing light intensity. Apparently, none of the *mPer* or *mCry* genes is necessary for the suppressing effect of light on general activity.

Rhythmicity

mPer2^{Brdm1} mice are rhythmic in LL, as previously found by Steinlechner et al. (2002). In this study we show that after loss of rhythmicity in DD exposure to L:D 12:12 is not required for *mPer2*^{Brdm1} mice to become rhythmic again. The rhythm is thus self-excitatory. Constant light either initiates the circadian oscillation, or the pacemaker regains control over its behavioral output.
Low levels of rhythmicity are generally associated with low activity (Aschoff 1960, Turek 1989). This holds also for all genotypes tested here, except for *mPer2*^{Brdm1} mice exposed to lower levels of constant light. Although *mPer2*^{Brdm1} mice are arrhythmic in DD, their activity level in DD is comparable to that in LD. In addition, arrhythmic *mPer2*^{Brdm1} mice in DD are much more active than rhythmic *mPer1*^{Brdm1} mice. Only when exposed to high LL light intensities, the SNR in *mPer2* mutants may be reduced by severely suppressed activity levels.

mPer1^{Brdm1} mice were least rhythmic in LL and showed least locomotor activity of all genotypes. In contrast to m*mPer2*^{Brdm1} mice, rhythmicity in *mPer1*^{Brdm1} mice is almost entirely lost in high intensity LL but recurs with decreasing light intensity. The low SNR in *mPer1*^{Brdm1} mice under exposure of bright light does not confirm the interpretation by Steinlechner et al. (2002a), that rhythmicity is sustained in *mPer1*^{Brdm1} in these conditions. In our study, wild type mice were most rhythmic in all conditions, but with a clear suppression in level of rhythmicity by high light intensities.
In the two-oscillator model as proposed by Daan et al. (2001) a distinct role for *mPer1* in the M- and for *mPer2* in the E-component of the circadian oscillator was suggested. These two oscillators were predicted to respond oppositely in velocity and hence phase when the circadian system is exposed to light. These opposing influences could possibly account for arrhythmicity in wild type mice in LL as suggested by Daan et al (2001). Mice, single mutant for *mPer1, mPer2, mCry1* or *mCry2*, or mice double mutant for *mPer1mCry1* or *mPer2mCry2* would then be exempted from these opposite forces in LL conditions and be expected to more readily preserve rhythmicity. This is not the case. Compared to *mPer1*^{Brdm1} and *mPer2*^{Brdm1}, wild type mice have a more stable rhythm that is less disturbed by increasing light intensity than *mPer1*^{Brdm1} mice. Wild type control mice are equally rhythmic as *mCry1*$^{-/-}$ or *mCry2*$^{-/-}$ mice in any condition.

Taken together, mutations of *mPer1* and *mPer2* have opposite effects on the influence of constant light on the circadian system, while the deletions of *mCry1* and *mCry2* cause opposite effects on circadian period independent of the light intensity. Deceleration of the circadian system by light in both *mCry1* and *mCry2* mutants is not in agreement with the predictions from the model, which therefore has to be modified.

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